Prostaglandins participate in the late phase of the vascular response to acetylcholine iontophoresis in humans

S. Durand, M. Tartas, P. Bouyé, A. Koïtka, J. L. Saumet and P. Abraham

UMR CNRS 6188, Laboratory of Physiology, University of Medicine, 49045 Angers cedex 01, France

The participation of prostaglandins (PGs) in the cutaneous vasodilatation to acetylcholine (ACh) applied via iontophoresis is under debate. Using laser Doppler flowmetry, we studied the long lasting effect (20 min) of iontophoretic application (30 s; 0.1 mA) of ACh on the human forearm. Experiments were repeated (1) using deionized water instead of ACh to test the effect of current application, (2) after scopolamine treatment to inhibit muscarinic cholinergic receptors, and (3) 2 h, 3 days and 10 days following inhibition of PG synthesis with aspirin or a placebo control. Cutaneous vascular conductance (CVC) was calculated at rest (CVC_{rest}), at peak vasodilatation in the first 5 min following ACh iontophoresis (CVC_{peak}), and 20 min after iontophoresis (CVC_{20}). The minimal CVC (CVC_{min}) following iontophoresis was also determined. Cutaneous response to ACh displayed a biphasic pattern with an early and transient peak (CVC_{peak}: 62 ± 8% of the maximal CVC induced by local heating (MVC)) followed by a long lasting slower vasodilatation (CVC_{min}: 44 ± 6; CVC_{20}: 56 ± 5%MVC). The current itself had no major effect. Scopolamine almost abolished both phases. The long lasting phase was aspirin sensitive but not the transient phase. At hour 2 post-aspirin, CVC_{peak} was 61 ± 10, CVC_{min} 26 ± 6 and CVC_{20} 29 ± 6%MVC. At day 3, CVC_{peak} was 53 ± 9, CVC_{min} 22 ± 3 and CVC_{20} 25 ± 4%MVC. At day 10, CVC_{peak} was 67 ± 10, CVC_{min} 47 ± 7 and CVC_{20} 50 ± 8%MVC. Placebo had no effect. We conclude that PGs participate in the vasodilator response following ACh iontophoresis. Previous non-steroidal anti-inflammatory drug treatments must be taken into account when studying the effect of ACh iontophoresis.

(Received 15 June 2004; accepted after revision 20 October 2004; first published online 21 October 2004)
Corresponding author P. Abraham: Laboratory of Vascular Investigations, University Hospital, 49033 Angers cedex, France. Email: piabraham@chu-angers.fr

Among the complications resulting from systemic diseases such as diabetes mellitus or hypertension are cutaneous vascular dysfunctions resulting from altered endothelial vasomotor function. Tests of the vascular effect of acetylcholine (ACh; an endothelium-dependent vasodilator) are often performed to evaluate the endothelium-dependent microvascular responses (Katz *et al.* 2001; Jagren *et al.* 2002). Controversies concerning the mechanisms of the ACh-induced cutaneous vasodilatation remain. A variety of vasomotor factors may be released following ACh action on the endothelium including endothelium derived hyperpolarizing factor, nitric oxide (NO) and some prostanoids. However, the exact mechanisms of ACh-induced cutaneous vasodilatation and the influence of these factors remain unresolved. Some investigators have suggested the participation of prostaglandins (PGs) in the ACh-dependent cutaneous vasodilatation (Khan *et al.* 1997; Noon *et al.* 1998), whereas others reported no role for PGs (Morris & Shore, 1996). This remains an important question since a role for PGs in the vasodilatation would mean that any use of non-steroidal anti-inflammatory drugs could confound interpretation of this test of endothelial function.

Berghoff *et al.* (2002) showed recently that the prostanoids did not significantly participate in the vasodilator effect of ACh. However they were involved in the axon-reflex response appearing in the area close to the site of ACh administration. They also proposed that the discrepancy in results found in the literature could be due to an unrecognized axon reflex within the stimulation area.

All of the aforementioned studies used iontophoresis to deliver ACh. However, different aspirin formulations and administration techniques were used to study the participation of prostanoids, making interpretations difficult. Also, analyses of the results were mainly based on peak vascular responses following ACh administration. None of the studies focused on the long-lasting cutaneous vasodilator effects of ACh. We believe that the study of

 DOI: 10.1113/jphysiol.2004.069997

the long-lasting vasodilator effects of ACh could provide essential information about the participation of PGs in this response. We hypothesized that the cutaneous vascular response to ACh applied by iontophoresis results from two parallel mechanisms, one of which would be aspirin sensitive. Using laser Doppler flowmetry (LDF) in humans, we first studied the long lasting effect (20 min) of iontophoretically applied ACh on cutaneous microcirculation and we tested the cholinergic specificity of the responses observed with scopolamine. To study the influence of prostanoids in the vascular response observed and to determine the origin of the prostanoids that may be involved, we also repeated iontophoretic administration of ACh 2 h, 3 days and 10 days following aspirin or placebo administration. These questions have important clinical implications regarding the evaluation of endothelial dysfunction using ACh and could provide important information about the potentially confounding long-lasting effect of aspirin treatment during such procedures.

Methods

Eight non-smoking healthy volunteers (3 females, 5 males) with no clinical signs of or risk factors for vascular disease (mean ± S.D.; 28 ± 6 years old; height: 171 ± 12 cm; weight: 65 ± 12 kg), participated in this study. Volunteers were not involved in regular exercise training and had not taken any medication during the 3 weeks prior to each experiment. A minimum of 1 week elapsed between each experiment. The study was institutionally approved and performed in accordance with the Declaration of Helsinki. Before their participation, all subjects were thoroughly informed of the methods and procedures and gave their written consent to participate.

Measurements

Experiments were performed in a quiet air-conditioned room (ambient temperature: 23 ± 1°C). Subjects rested supine for 15 min before the experiment was started.

Iontophoresis and measurement of skin blood flow (SkBF) occurred on the volar aspect of the forearm. Iontophoresis is a non-invasive technique that allows transcutaneous delivery of polarized drugs via the application of a continuous low intensity current. We used the PeriIont PF 382, Micropharmacology System from Perimed (Stockholm, Sweden) associated with laser Doppler flowmeters (PeriFlux PF4001 and 5000, Perimed) and temperature regulated heating system (PeriTemp PF4005 and 5000, Perimed) to allow for current application, SkBF recording and local heating, respectively. Technical characteristics of these devices can be found in previous reports (Durand *et al.* 2002*a*,*b*). The 'active' electrode of the system was fixed to the skin and connected to the anode of the regulated 9 V intensity regulated current supplier. The sponge of this electrode was wet with 0.2 ml of ACh (2% in deionized water). The cathode was connected to a reference Ag–AgCl electrode placed nearby. In this study, the total charge of anodal current applied was 3 mC (application time: 30 s; intensity: 0.10 mA). Subjects reported no pain during the iontophoresis procedure. Temperature for local heating at the end of the experiments was set to 44°C to allow maximal vasodilatation (Taylor *et al.* 1984; Johnson *et al.* 1986; Saumet *et al.* 1998). Another LDF probe was used as a reference to confirm the absence of response to the current application at an unstimulated site 5 cm adjacent to the 'active' electrode. Local cutaneous temperature was measured using a surface thermocouple probe connected to an electronic thermometer (BAT-12, Physitemp Instruments Inc., Clifton, NJ, USA). The thermocouple probe was positioned 5 cm from the LDF probes.

Systemic blood pressure was monitored using a Finapres 2350 (Ohmeda, Englewood, CO, USA) positioned on the 2nd or 3rd finger of the hand contralateral to the sites of SkBF measurements.

Protocols

For all the protocols in this study, recordings consisted of a 2-min rest period followed by the iontophoresis period (30 s). A 20-min recovery period was then followed by local heating for 25 min. As a result, the total duration of each recording was 47.5 min

Protocol 1. Evaluation of non-specific response to anodal current application and effect of ACh iontophoresis on SkBF. The use of continuous monopolar current (i.e. galvanic current) during iontophoresis could lead to a non-specific vasodilatation independent of the effect of the drug diffused (Grossman *et al.* 1995; Berliner, 1997; Durand *et al.* 2002*a*). We assessed the possible role of this non-specific vasodilatation in the response observed following ACh iontophoresis by performing iontophoresis with deionized water alone as a vehicle. Deionized water has been previously used to evaluate the non-specific response to current application (Khan *et al.* 1997; Durand *et al.* 2002*a*; Özbebit *et al.* 2004). We then used ACh, 2% in deionized water, to assess the cutaneous microvascular response to ACh iontophoresis.

Protocol 2. Sensitivity of ACh-induced vasodilatation to muscarinic receptor inhibition. ACh causes vasodilatation via interaction with muscarinic receptors. To demonstrate the specificity of the response to muscarinic receptors in our model, ACh was applied via iontophoresis at two different sites. One site (site S) was treated with scopolamine in the form of a Scopoderm® patch (Novartis, Rueil-Malmaison, France) for 2 h prior to the start of the experiment to locally inhibit muscarinic receptors while the other site (site C) served as a control. The skin was

carefully washed before positioning of the iontophoretic electrodes and laser Doppler probes.

Protocol 3. Sensitivity of ACh-induced vasodilatation to aspirin. Each subject randomly participated in two experiments separated by a minimum of 3 weeks, one under aspirin pretreatment and the other under placebo pretreatment. 1-g aspirin (Aspégic®, SanofiSynthelabo, Paris, France) was dissolved in 125 ml of orange juice in order to disguise the taste and appearance of the aspirin, whereas nothing was added to the orange juice in the placebo experiments. Two hours prior to the beginning of the experiment, subjects drank the orange juice, blinded to the presence or absence of aspirin in the glass. The experiment was repeated 3 and 10 days following the intake of the drink.

Data analysis

Analog output from all measured data was recorded on a computer via a 16-bit analog to digital converter (Biopac Systems, Inc., Goleta, CA, USA) with a sample rate of 3 Hz. The LDF signal was expressed in arbitrary units (AU). Due to instantaneous variability of the LDF signal resulting from vasomotion, individual results were averaged over 5-s intervals throughout each experiment. To index active changes in the cutaneous vasculature, vasodilatation was assessed as cutaneous vascular conductance (CVC) and calculated as the ratio of LDF to mean arterial pressure for each 5-s interval. Maximal CVC response to local heating (MVC) was calculated from each experiment from the average of CVC values observed over the last minute of the heating period. CVC values were normalized and presented as a percentage of the MVC (%MVC).

For data analysis and interpretation we defined the following points: CVC_{rest} was the last 5-s interval recorded before the current application; CVC_{peak} was the maximal value recorded during, or in the 5 min following, current application; CVC_{min} was the minimal CVC values observed following current application and CVC_{20} was the value recorded 20 min after the current application, just prior to local heating. An example of the determination of these key points is presented in Fig. 1. All time references reported in the Results are from the start of the current application and were estimated for a protocol from the mean result, not from the mean of the individual times.

Student's *t* test was used to compare CVC data within a protocol. The influence of aspirin and placebo treatment on CVC (protocol 3) was assessed by two-way repeated measures ANOVA (Prism 2.01, GraphPad Software Inc., San Diego, CA, USA). Data are expressed as means ± S.E.M. $P < 0.05$ was considered significant in all statistical analyses. N.S. is reported in the text for statistically non-significant results.

Table 1. Effect of anodal iontophoresis of acetylcholine, 2% in deionized water, and deionized water alone (DW) on cutaneous microcirculation measured by laser Doppler flowmetry

	ACh	DW
CVC_{rest}	11 ± 2	11 ± 3
CVC_{peak}	62 ± 8†	14 ± 3
CVC_{min}	44 ± 6†*	9 ± 2
CVC_{20}	56 ± 5†‡	18 ± 4†‡

Anodal current application: 30 s; 0.1 mA. Results are expressed as a percentage of the maximal cutaneous vascular conductance observed after a 25-min local heating period (44°C). †$P < 0.05$ compared to CVC_{rest}. *$P < 0.05$ *versus* CVC_{peak}. ‡$P < 0.05$ *versus* CVC_{min}.

Results

In all experiments, compared with starting values, no significant changes were observed in mean blood pressure, local skin temperature or skin blood flow at the control probe.

Protocol 1. Evaluation of the non-specific response to anodal current application and effect of ACh iontophoresis on SkBF

Effect of anodal current application on SkBF. CVC_{rest} was 11 ± 3%MVC. Iontophoresis of deionized water did not result in any immediate vasodilatation (Table 1). CVC_{min} was 9 ± 2%MVC (NS *versus* CVC_{rest}) at min 1.0 and CVC_{peak} was 14 ± 3%MVC at min 5.1 (NS *versus* CVC_{rest}). However, a slow drift in CVC could be observed during the experiment with CVC_{20} reaching 18 ± 4%MVC ($P < 0.05$ *versus* CVC_{rest} and *versus* CVC_{min}).

Effect of ACh iontophoresis on SkBF. A typical recording of SkBF following ACh iontophoresis in one subject is presented in Fig. 2. The mean response to ACh iontophoresis is shown in Fig. 1. CVC_{rest} was 11 ± 2%MVC. Iontophoresis of ACh induced a rapid and large vasodilatation. This cutaneous vasodilatation displays a biphasic pattern with an early peak (phase 1 of the vasodilatation) and a long lasting slower vasodilatation (phase 2 of the vasodilatation). At the end of the current application (i.e. end of ACh administration), CVC reached 23 ± 4%MVC ($P < 0.01$ *versus* CVC_{rest}). It was 38 ± 6 and 59 ± 7%MVC 15 s and 1 min later, respectively. CVC_{peak} (62 ± 8%MVC, $P < 0.001$ *versus* CVC_{rest}) was attained at 2.2 min following the end of iontophoresis. CVC then transiently decreased (end of phase 1). CVC_{min} reached 44 ± 6%MVC ($P < 0.001$ *versus* CVC_{rest} and $P < 0.05$ *versus* CVC_{peak}) at min 9.0. A progressive and slow vasodilatation followed (phase 2) and CVC_{20} was 56 ± 5%MVC (NS *versus* CVC_{peak}; $P < 0.05$ *versus* CVC_{min} and CVC_{rest}).

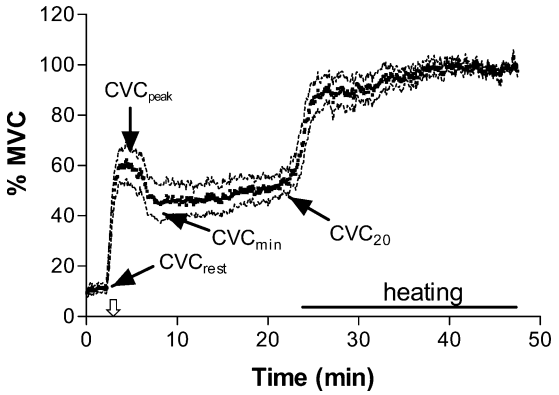

Figure 1. Illustration of the different points analysed on the laser Doppler flowmetry signals
Data presented are the mean response ± S.E.M. ($n = 8$) observed before, during and after an ACh iontophoretic administration (30 s; 0.1 mA) at the forearm skin level. Results are expressed as a percentage of the maximal cutaneous vascular conductance (%MVC) observed after a 25-min local heating period (44°C). The white arrow indicates the period of ACh iontophoresis. Note the biphasic response to ACh iontophoresis with an early peak in CVC followed by a slow and long-lasting vasodilatation.

Protocol 2. Sensitivity of ACh-induced vasodilatation to muscarinic receptor inhibition

No differences in CVC_{rest} were observed between site S and site C (12 ± 2 *versus* 12 ± 3%MVC, respectively). Scopolamine profoundly blunted the ACh-dependent vasodilatation. There was a significant difference in CVC_{peak} between site S and site C. CVC_{peak} at site C reached 57 ± 14%MVC but was only 20 ± 6%MVC at site S (NS *versus* CVC_{rest} and $P < 0.05$ *versus* CVC_{peak} at site C). CVC remained stable at site S in the 20 min following the end of the iontophoresis, whereas the late vasodilatation was still observed at site C. At min 20, CVC_{20} was 40 ± 12%MVC at site C ($P < 0.05$ *versus* CVC_{rest} and CVC_{peak}) and 18 ± 5%MVC at site S (NS *versus* CVC_{rest} and CVC_{peak}; Fig. 3).

Protocol 3. Sensitivity of ACh-induced vasodilatation to aspirin

The effects of aspirin and placebo pretreatments on the vascular response to ACh iontophoresis are presented in Fig. 4 and Table 2.

Placebo treatment did not affect the response to iontophoretic ACh administration at all time points. CVC_{peak} was significantly higher than CVC_{rest}. Then CVC transiently decreased with CVC_{min} significantly different from CVC_{peak}. CVC_{20} was not different from CVC_{peak} but different from CVC_{min}.

Aspirin pretreatment did not modify the first phase of the vasodilatation induced by ACh iontophoresis. No differences were observed in CVC_{peak} under aspirin or

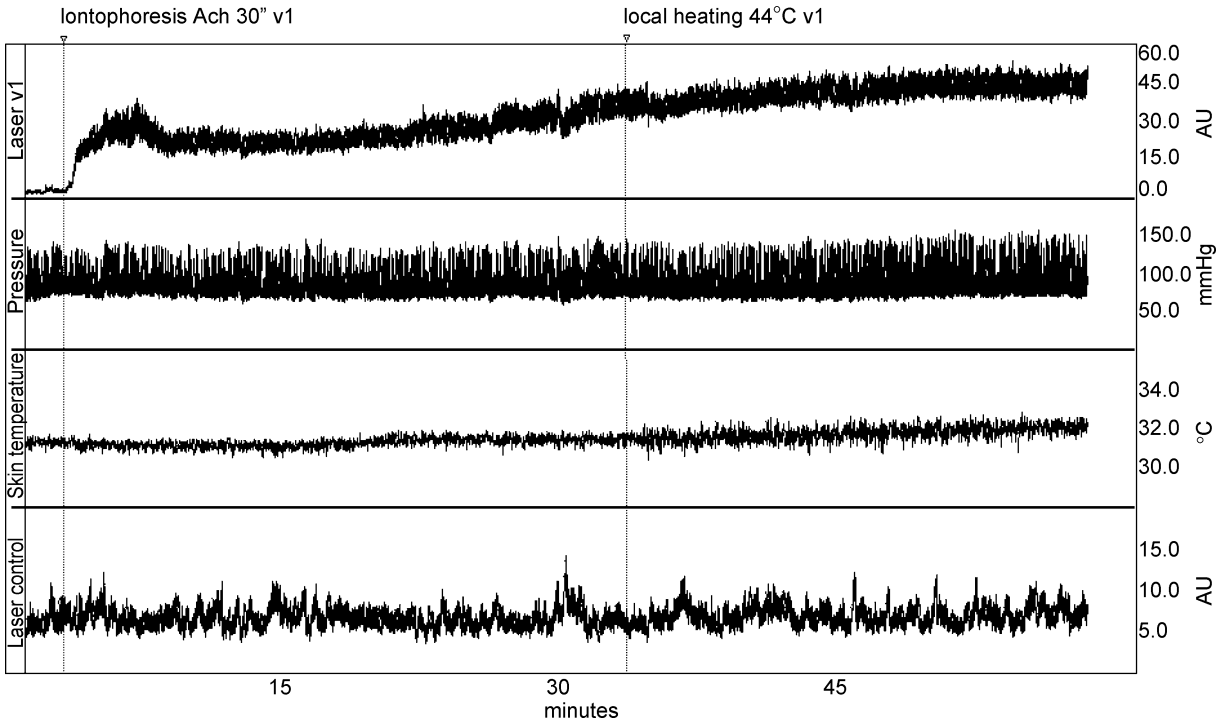

Figure 2. Typical cutaneous vascular response to the iontophoretic application of ACh (30 s; 0.1 mA) in forearm skin
From top to bottom, recordings are: SkBF under the 'active' electrode where the ACh iontophoresis was performed (Laser v1); systemic blood pressure monitored via Finapres (Pressure); local cutaneous temperature in the area close to the iontophoresis site (Skin Temperature); and SkBF at an unstimulated control site (Laser control).

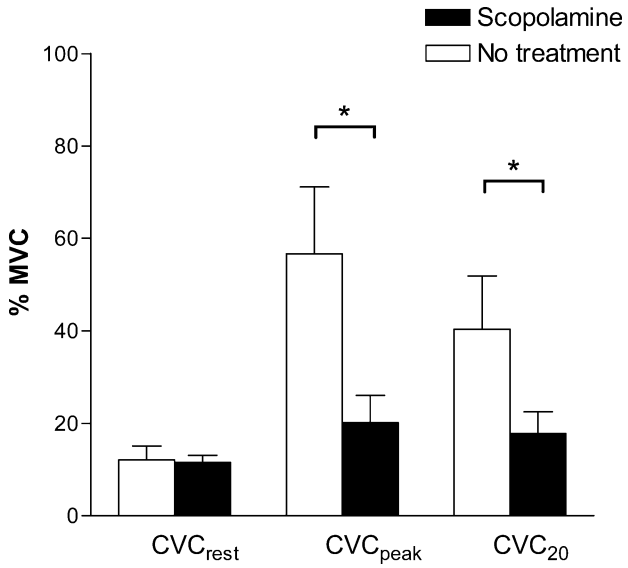

Figure 3. Effect of muscarinic receptor inhibition by scopolamine on the cutaneous vasodilatation induced by iontophoretic administration of ACh
Results are expressed as a percentage of the maximal cutaneous vascular conductance (%MVC) observed after a 25-min local heating period (44°C).

placebo treatment at any of the time points we studied (hour 2, day 3 or day 10). The second phase of the vasodilator response was clearly affected suggesting PG involvement. Two hours after aspirin intake, this phase 2 vasodilatation was abolished. A plateau was observed at a level higher than CVC_{rest} but lower than CVC_{peak}. CVC_{min} was not different from CVC_{20}. In contrast to protocol 1, CVC_{20} was lower than CVC_{peak} and it was also lower than CVC_{20} at 2 h following placebo treatment. A progressive recovery of the slow vasodilatation occurred in the 10 days following aspirin treatment. On day 3, following the early peak vasodilatation, the plateau was changed to a progressive fall in CVC throughout the recovery period. CVC_{min} was not different from CVC_{20} and CVC_{20} was lower than CVC_{peak}. Compared to placebo treatment, CVC_{20} was diminished after aspirin ($P < 0.05$ placebo *versus* aspirin). At day 10, the phase 2 vasodilatation returned, such that CVC_{20} was not different from CVC_{peak}. However, despite the similarities with the responses observed in protocol 1 and under placebo treatment, the time course of this slow and long lasting vasodilatation was slightly different from what was observed in the previous protocols, resulting in CVC_{min}

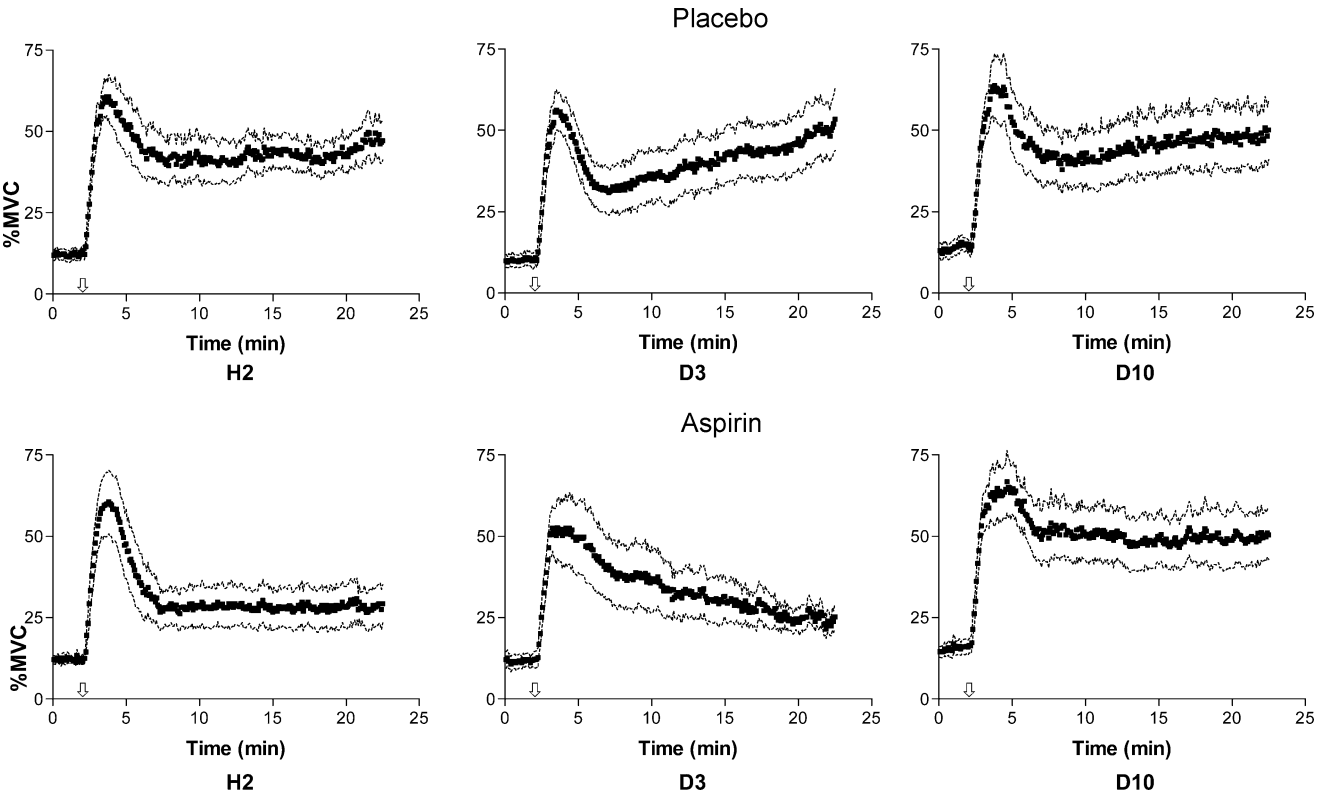

Figure 4. Mean cutaneous vascular conductance (CVC) observed before, during and 20 min following the ACh iontophoresis (30 s; 0.1 mA), at different intervals from aspirin (bottom graphs) or placebo (top graphs) treatment: hour 2 (H2), day 3 (D3) and day 10 (D10)
Results are expressed as a percentage of the maximal cutaneous vascular conductance (%MVC) observed after a 25-min local heating period (44°C). The white arrow indicates the period of ACh iontophoresis. The heating period is not presented, to simplify the graphs.

Table 2. Mean CVC_{rest}, CVC_{peak}, CVC_{min} and CVC_{20} observed following ACh iontophoresis 2 h (H2), 3 days (D3) or 10 days (D10) after aspirin or placebo treatment

	Aspirin treatment			Placebo treatment		
	H2	D3	D10	H2	D3	D10
CVC_{rest}	12 ± 1	12 ± 2	16 ± 2	12 ± 2	10 ± 2	15 ± 2
CVC_{peak}	61 ± 10†	53 ± 9†	67 ± 10†	61 ± 6†	56 ± 6†	64 ± 10†
CVC_{min}	26 ± 6†*	22 ± 3†*	47 ± 7†*	39 ± 7†*	31 ± 7†*	38 ± 7†*
CVC_{20}	29 ± 6†*	25 ± 4†*	50 ± 8†	47 ± 6†‡	†‡53 ± 10	49 ± 8†‡

Results are expressed as a percentage of the maximal cutaneous vascular conductance observed after a 25-min local heating period (44°C). †$P < 0.05$ *versus* CVC_{rest}. *$P < 0.05$ *versus* CVC_{peak}. ‡$P < 0.05$ *versus* CVC_{min}. Note that at D10 after aspirin treatment, a response almost similar to the one observed during the placebo experiments can be observed.

being lower than CVC_{peak} but not different from CVC_{20} ($P = 0.2$).

Discussion

The long lasting effect of iontophoretic administration of ACh in the skin has not been well described. We report in the present study that the cutaneous vasodilator response to iontophoretic administration of ACh at the forearm displays a biphasic pattern and that PGs are involved in the second phase of this cutaneous microvascular response.

In the present study, the vascular response to 30-s iontophoretic administration of ACh (total electrical charge = 3 mC) at the cutaneous level included an early (within 5 min following the start of current application), short lasting, peak vasodilatation followed by a late, slower prolonged vasodilatation. This vasodilatation was still present after 20 min and resulted in CVC levels in the same range of the peak vasodilatation observed in the early vasodilator response. Iontophoresis has been previously reported to induce a non-specific long-lived cutaneous vasodilatation due to the use of the current itself (Grossmann *et al.* 1995; Durand *et al.* 2002*a*,*b*). ACh-induced vasodilatation is based on the effect of ACh on cutaneous M2 muscarinic receptors. Involvement of these receptors in the mechanism of the non-specific vasodilatation is not known. However after local cutaneous inhibition of the muscarinic receptors, no significant vasodilatation was observed following ACh iontophoresis, suggesting that both phases of the cutaneous vasodilatation were specifically related to ACh. The slow drift of SkBF accompanying the iontophoresis of deionized water led to a very limited vasodilatation (from 13 ± 3 to 18 ± 4%MVC). This also suggests that there was no major influence of the technique itself.

Aspirin is known to inhibit the synthesis of prostanoids through an irreversible blockade of cyclooxygenase (Vane, 1971). The early phase of the ACh-induced vasodilatation was insensitive to aspirin treatment. No change in the peak vasodilatation was observed. This observation is consistent with the results of Berghoff *et al.* (2002) and Morris & Shore (1996) but differs with the previous reports of Noon *et al.* (1998) and Khan *et al.* (1997). These latter authors observed a significant decrease in the vascular response to ACh applied via iontophoresis following aspirin treatment. All of the aforementioned (Morris & Shore, 1996; Khan *et al.* 1997; Noon *et al.* 1998; Berghoff *et al.* 2002) performed dose–response studies to investigate the participation of PG in the response to ACh iontophoresis, focusing only on the peak response observed shortly after ACh iontophoresis. Morris & Shore (1996), using a laser imager, recorded the responses at 0, 60 and 120 s after the iontophoresis; Khan *et al.* (1997) recorded changes in flux for 4 min between each dose applied; Berghoff *et al.* (2002) applied the current every 3 min while Noon *et al.* (1998) analysed the response observed within the 2 min maximum following iontophoresis. Reasons for discrepancies in their conclusions are unknown but could be attributed to differences in the iontophoretic protocol, the maximal current charge used (from 4 to 56 mC), and the method of aspirin administration.

Vallance *et al.* (1989*a*,*b*) concluded that NO participates in the ACh-mediated vasodilatation in human arteries and veins *in vivo*. The early peak vasodilator response could reflect the release of NO due to the action of ACh on the endothelial M2 muscarinic receptors. Those receptors can activate constitutive endothelial NO synthases via a G protein mechanism leading to the production of NO. NO has a short half-life and is one of the major and most powerful vasodilator factors present in humans. Some *in vitro* studies suggest that salicylate treatment could affect NO production in human platelets (O'Kane *et al.* 2003) but aspirin does not have a significant effect on NO bioavailability in endothelial cells (Madajka *et al.* 2003) and we did not observe any significant difference in CVC_{peak} after placebo or aspirin treatment. This supports a role for NO in

ACh-mediated vasodilatation in human skin. However, *in vitro* studies on isolated human and animal vessels reported the participation of a non-NO, non-prostanoid endothelium-dependent hyperpolarizing mechanism in ACh-mediated vasodilatation (Buus *et al.* 2000; Hoepfl *et al.* 2002). Further research is necessary to confirm NO as the major factor responsible for the cutaneous response observed.

The late phase of the response was a prolonged vasodilatation which could be due to a neurogenic inflammation and a local axon reflex within the stimulation area, as suggested by Berghoff *et al.* (2002). ACh can excite unmyelinated C fibres (Douglas & Ritchie, 1960) and induce an axon reflex leading to prolonged vasodilatation in the close area of administration (Berghoff *et al.* 2002). Özbebit *et al.* (2004), using mathematical curve analysis, also concluded that the SkBF response to ACh iontophoresis was the sum of two components, one of which (the late component) was abolished after local anaesthesia. This provides further support for the participation of unmyelinated C fibres in the ACh-mediated response. Unmyelinated C fibres contain pools of vasomotor products such as calcitonin gene related peptide, substance P, or PG. It is possible that ACh induces an excitation of C fibres leading to the release of vasomotor products and to the progressive vasodilatation observed following the peak vasodilatation.

We observed that the phase 2 (late) vasodilatation was aspirin sensitive, suggesting that PGs are involved. PGs, whatever their origins, could act as a direct vasodilator agent on the cutaneous vessels. PGI2 (prostacyclin), for example, exhibits these vasorelaxing properties. They could also act as sensitizing agent on C fibres since specific PGs such as the PGE2 were shown to sensitize those fibres (Kindgen Milles, 1995; Minami *et al.* 1999). A combination of both mechanisms is also possible. Further investigations will be necessary to determine the exact mechanism by which PGs participate in the late phase of ACh-mediated vasodilatation.

If PGs are involved in the vasodilatation following ACh iontophoresis, the origin of the PGs remains unknown. Aspirin irreversibly blocks cyclooxygenase (COX). PG synthesis is restored when COX is resynthesized by nucleated cells. Restoration of COX is variable according to the tissue. For example, *in vitro*, COX in smooth muscle is restored in 3 h whereas, in endothelial cells, synthesis is altered for up to 24 h (Hla & Bailey, 1989). In an effort to determine the origin of the PGs participating in the ACh-induced vasodilatation, we followed the SkBF response to ACh iontophoresis 2 h, 3 days and 10 days after a single oral dose of 1 g aspirin. Such a dose blocks the whole pool of COX in the human body (Patrono *et al.* 1998). We observed that the return of the late phase of the vasodilatation following COX inhibition was progressive. A significant inhibition was still observed 3 days after aspirin intake at a time when the endothelial and smooth muscle function COX function should be entirely restored (Hla & Bailey, 1989). This suggests that part of the pool of PGs necessary to obtain the late phase of the vasodilatation to ACh iontophoresis is not of endothelial or smooth muscle cell origin. One possible origin of the PGs may be the nerve (C fibre). In support of this idea, aspirin has been shown to have an inhibitory effect on type 1 vanilloid receptor (VR1), a specific receptor of the non-myelinated C fibre (Szallasi & Blumberg, 1999). In these nucleated cells, due to the necessary axonal transport of molecules, a delay is likely to exist between the synthesis of proteins in the nucleus and the bioavailability at the nerve terminal. However, there is no available information about the time necessary for the VR1 or PG synthesis in the nucleus of C fibres and the delay for the axonal transport of the synthesized COX and/or VR1.

Aspirin may also interfere with the expression of inflammatory factors such as the nuclear factor-kappa B (Kopp & Ghosh, 1994) or interleukin-4 (Cianferoni *et al.* 2001). Since the non-specific vasodilatation could be due to a neurogenic inflammation from the current application (Berliner, 1997), these products may have been activated during our experiments. However, the possible influence of these factors during iontophoresis experiments remains unknown (Durand *et al.* 2002*c*). Could platelets be involved? These cells being devoid of synthesis ability, up to 10 days are necessary to restore the pool of platelet PGs after irreversible blockade of COX through the remaining life of the platelets with 1-g aspirin treatment (Patrono *et al.* 1998). At day 3, approximately 70% of platelets are still blocked. Further studies are needed to test these possibilities.

Finally, ACh and/or aspirin could have an effect on some other undescribed elements participating directly or indirectly in the vascular response to ACh iontophoresis. Consistent with the suggestion of Berghoff *et al.* (2002), we propose that ACh administered via iontophoresis, has a dual effect at the microvascular level. ACh could act on endothelial receptors to induce the release of vasodilator mediators responsible for the early vasodilatation. This step would be aspirin insensitive. It could also directly or indirectly excite primary afferent nerve fibres to elicit a prolonged long lasting vasodilator phenomenon (neurogenic inflammation). Contrary to the early phase, this late phase seems to be aspirin sensitive, relying in part on PG.

In summary, the present study results in new considerations about the vascular response to iontophoretically applied ACh and has important clinical implications. This response is biphasic with an early peak and a second, slower vasodilatation, both with different kinetics and likely different mediators. We have demonstrated that PGs are involved in the late

phase of the vasodilatation, but not in the early phase. ACh iontophoresis is regularly performed in clinical and scientific settings to assess the endothelium-dependent vasodilatation. When exploring the vascular effect of ACh, the following recommendations should be followed: (1) observations should be of adequate time to monitor both the first phase and the second phase of the ACh-mediated cutaneous vasodilatation, and (2) anti-inflammatory drug treatment, specifically aspirin, should be monitored prior to testing, with testing occurring at a minimum of 3 days or longer following anti-inflammatory drug administration.

References

Berghoff M, Kathpal M, Kilo S, Hilz MJ & Freeman R (2002). Vascular and neural mechanisms of ACh-mediated vasodilation in the forearm cutaneous microcirculation. *J Appl Physiol* **92**, 780–788.

Berliner MN (1997). Skin microcirculation during tapwater iontophoresis in humans: Cathode stimulates more than anode. *Microvasc Res* **54**, 74–80.

Buus NH, Simonsen U, Pilegaard HK & Mulvany MJ (2000). Nitric oxide, prostanoid and non NO, non prostanoid involvement in acetylcholine relaxation of isolated human small arteries. *Br J Pharmacol* **129**, 184–192.

Cianferoni A, Schroeder JT, Kim J, Schmidt JW, Lichtenstein LM, Georas SN & Casolaro V (2001). Selective inhibition of interleukine-4 gene expression in human T cells by aspirin. *Blood* **97**, 1742–1749.

Douglas WW & Ritchie JM (1960). The excitatory action of acetylcholine on cutaneous non-myelinated fibres. *J Physiol* **150**, 501–514.

Durand S, Fromy B, Bouyé P, Saumet JL & Abraham P (2002*a*). Current-induced vasodilation during water iontophoresis (5 min, 0.10 mA) is delayed from current onset and involves aspirin-sensitive mechanisms. *J Vasc Res* **39**, 59–71.

Durand S, Fromy B, Bouyé P, Saumet JL & Abraham P (2002*b*). Vasodilatation to repeated anodal current applications in the human skin relies on aspirin-sensitive mechanisms. *J Physiol* **540**, 261–269.

Durand S, Fromy B, Koïtka A, Tartas M, Saumet JL & Abraham P (2002*c*). Oral single high-dose aspirin results in a long lived inhibition of anodal current induced vasodilatation. *Br J Pharmacol* **137**, 384–390.

Grossmann MG, Jamieson MJ, Kellogg DL Jr, Kosiba WA, Pergola PE, Crandall CG & Shepherd AMM (1995). The effect of iontophoresis on the cutaneous vasculature: evidence for current-induced hyperemia. *Microvasc Res* **50**, 444–452.

Hla TT & Bailey JM (1989). Differential recovery of prostacyclin synthesis in cultured vascular endothelial vs. smooth muscle cells after inactivation of cyclooxygenase with aspirin. *Prostaglandins, Leukot Essent Fatty Acids* **36**, 175–184.

Hoepfl B, Rodenwaldt B, Pohl U & De Witt C (2002). EDHF, but not NO or prostaglandins, is critical to evoke a conducted dilation upon ACh in hamster arterioles. *Am J Physiol* **283**, H996–H1004.

Jagren C, Gazelius B, Ihrman-Sandal C, Lindblad LE & Ostergren J (2002). Skin microvascular dilatation response to acetylcholine and sodium nitroprusside in peripheral arterial disease. *Clin Physiol Funct Imaging* **22**, 370–374.

Johnson JM, O'Leary D, Taylor WF & Kosiba W (1986). Effect of local warming on forearm reactive hyperaemia. *Clin Physiol* **6**, 337–346.

Katz A, Ekberg K, Johansson BL & Wahren J (2001). Diminished skin blood flow in type 1 diabetes: evidence for non-endothelium-dependent dysfunction. *Clin Sci (London)* **101**, 59–64.

Khan F, Davidson NC, Littleford RC, Litchfield SJ, Struthers AD & Belch JJ (1997). Cutaneous vascular responses to acetylcholine are mediated by a prostanoid-dependent mechanism in man. *Vasc Med* **2**, 82–86.

Kindgen Milles D (1995). Effects of prostaglandin E2 on the intensity of bradykinin-evoked pain from skin and veins of humans. *Eur J Pharmacol* **294**, 491–496.

Kopp E & Ghosh S (1994). Inhibition of NF-kappa B by sodium salycilate and aspirin. *Science* **265**, 256–259.

Madajka M, Korda M, White J & Malinski T (2003). Effect of aspirin on constitutive nitric oxide synthase and the bioavailability of. *Thromb Res* **110**, 317–321.

Minami T, Okuda-Ashitaka E, Hori Y, Sakuma S, Sugimoto T, Sakimura K, Mishina M & Ito S (1999). Involvement of primary afferents c-fibres in touch evoked pain (allodynia) induced by prostaglandin E2. *Eur J Neurosciences* **11**, 1849–1856.

Morris SJ & Shore AC (1996). Skin blood flow responses to the iontophoresis of acetylcholine and sodium nitroprusside in man: possible mechanisms. *J Physiol* **496**, 531–542.

Noon JP, Walker BR, Hand MF & Webb DJ (1998). Studies with iontophoretic administration of drugs to human vessels in vivo: cholinergic vasodilatation is mediated by prostanoids rather than nitric oxide. *Br J Clin Pharmacol* **45**, 545–550.

O'Kane PD, Queen LR, Ji Y, Reebye V, Stratton P, Jackson G & Ferro A (2003). Aspirin modifies nitric oxide synthase activity in platelets: effects of acute versus chronic aspirin treatment. *Cardiovasc Res* **59**, 152–159.

Özbebit FY, Esen F, Güleç S & Esen H (2004). Evaluation of forearm microvascular blood flow regulation by laser Doppler flowmetry, iontophoresis, and curve analysis: contribution of axon reflex. *Microvasc Res* **67**, 207–214.

Patrono C, Coller B, Dalen JE, Fuster V, Gent M, Harker LA, Hirsh J & Roth G (1998). Platelet-active drugs: the relationships among dose, effectiveness and side effects. *Chest* **114**, 470S–488S.

Saumet JL, Abraham P & Jardel A (1998). Cutaneous vasodilation induced by local warming, sodium nitroprusside, and bretylium iontophoresis on the hand. *Microvasc Res* **56**, 212–217.

Szallasi A & Blumberg PM (1999). Vanilloid (capsaicin) receptor and mechanisms. *Pharmacol Rev* **51**, 159–211.

Taylor WF, Johnson JM, O'Leary D & Park MK (1984). Effect of high local temperature on reflex cutaneous vasodilation. *J Appl Physiol* **57**, 191–196.

Vallance P, Collier J & Moncada S (1989*a*). Effects of endothelium derived nitric oxide on peripheral arterial tone in man. *Lancet* **2**, 997–1000.

Vallance P, Collier J & Moncada S (1989b). Nitric oxide synthetised from 1-arginine mediates endothelium dependent vasodilatation in human veins in vivo. *Cardiovasc Res* **23**, 1053–1057.

Vane JR (1971). Inhibition of prostaglandin synthesis as a mechanism of action for the aspirin-like drugs. *Nat New Biol* **231**, 232–235.

Acknowledgements

The authors gratefully acknowledge Dr Scott Davis (USA) and Dr Nisha Charkoudian (USA) for reviewing the English. S.D. is supported by the Fondation Electricité de France. This study was promoted by the CHU d'Angers (PHRC 2001) with the help of the DRDJS des Pays de la Loire.